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On the Phylogenetic Position of *Cystodium*: It's Not a Tree Fern – It's a Polypod!

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ABSTRACT.—The phylogenetic position of *Cystodium* J. Sm. is studied here for the first time using DNA sequence data. Based on a broad sampling of leptosporangiate ferns and two plastid genes (*rbcL* and *atpB*), we show that *Cystodium* does not belong to the tree fern family Dicksoniaceae, as previously thought. Our results strongly support including *Cystodium* within the large polypod clade, and suggest its close relationship to the species-poor grade taxa at the base of the polypod topology (*Sphenomeris* and *Lonchitis*, or *Saccoloma* in this study). Further studies, with an expanded taxon sampling within polypods, are needed to fully understand the more precise phylogenetic relationships of *Cystodium*.

Cystodium J. Sm., with its single species *C. sorbifolium* (Sm.) J. Sm., has traditionally been included in Dicksoniaceae (Christensen, 1938; Pichi Sermolli, 1977; Tryon and Tryon, 1982; Kramer, 1990; Stevenson and Loconte, 1996) and, more specifically, has been considered to be closely related to *Dicksonia* L'Hér. (Hooker, 1844; Copeland, 1947; Holttum and Sen, 1961). *Cystodium* grows in lowland rainforests from Borneo to New Guinea and adjacent islands such as the Bismarck and Louisiade Archipelagos, and the Solomon Islands (Holttum, 1963; Croft, 1986; Kramer, 1990). It has a creeping, hairy, dictyostelic rhizome with large, bipinnate leaves that are two meters or more in length (Croft, 1986). The terminal sori are covered by a true indusium

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and an adaxial, false indusium, a modified part of the leaf (Croft, 1986). This indusium type has been highlighted as an important feature that unites *Cystodium* with *Dicksonia*, *Thyrsopteris* Kunze, *Culcita* C. Presl, *Calochlaena* (Maxon) Turner & White, and *Cibotium* Kaulf. within Dicksoniaceae (Kramer, 1990).

Cystodium was not included in any of the large-scale molecular phylogenetic studies in the last decade that focused on the relationships of monilophytes and leptosporangiate ferns (Hasebe *et al.*, 1994, 1995; Pryer *et al.*, 1995; Rothwell, 1999; Pryer *et al.*, 2001, 2004; Schneider *et al.*, 2004; Wikström and Pryer, 2005). In several of these studies, Dicksoniaceae, represented by one or more of its genera (never *Cystodium*), was consistently found to be closely related to Cyatheaceae in a clade termed the "tree ferns", where most of the taxa are arborescent. When more than one representative of Dicksoniaceae was included in these DNA studies, the monophyly of the family was often called into question (Hasebe *et al.*, 1994, 1995; Wolf *et al.*, 1999; Pryer *et al.*, 2004). In a strictly morphological cladistic analysis by Stevenson and Loconte (1996), where *Cystodium* was included along with other dicksoniaceous ferns, Dicksoniaceae was recovered as monophyletic. However, including *Cystodium* in Dicksoniaceae, has been challenged based on differences in its stipe anatomy (Nishida, 1984; Croft, 1986) and spore morphology (Gastony, 1981). Croft (1986) even considered *Cystodium* to be so distinctive from other dicksonioid genera that it merited its own family, Cystodiaceae. Here we use DNA sequence data from two plastid genes (*rbcL* and *atpB*) to investigate the phylogenetic relationship of *Cystodium* to other leptosporangiate ferns, and specifically to address whether it belongs within tree ferns.

Materials and Methods

Taxon sampling, DNA isolation, amplification, sequencing, and sequence alignment.—Total DNA of *Cystodium sorbifolium* was extracted using the DNeasy plant mini kit from Qiagen (Valencia, California, USA). The voucher specimen is *Christensen 1529* (S) (record no. 2498 in Fern DNA database at http://www.biology.duke.edu/pryerlab/ferndb). The *rbcL* and *atpB* genes from the plastid genome were amplified using the polymerase chain reaction (PCR), following standard protocols. PCR products were cleaned using the Montage PCR cleanup kit (Millipore, Billerica, Massachusetts, USA) according to the manufacturer's protocol. Sequencing reactions were carried out for both strands of the purified PCR products using Big Dye Terminator Cycle Sequencing reagents (Applied Biosystems, Foster City, California, USA). For information on amplification and sequencing primers, see Table 1. All sequencing reactions were processed using either ABI 3700 or ABI 3730XL automated sequencers (Applied Biosystems). Sequence fragments were assembled and edited using Sequencher version 4.2.2 (Gene Codes, Ann Arbor, Michigan, USA). The corrected consensus sequences were aligned manually using MacClade version 4.07b13 (Maddison and Maddison, 2005). The *rbcL* and *atpB* Genbank numbers for *C. sorbifolium* are AM184111 and

TABLE 1. Primers used for amplifying and sequencing *rbcL* and *atpB* from *Cystodium sorbifolium*.

Primer	Sequence (5′ to 3′)	References
rbcL		
ESRBCL1F	ATGTCACCACAAACGGAGACTAAAGC	Korall *et al.*, 2006
ES645F	AGAYCGTTTCYTATTYGTAGCAGAAGC	Korall *et al.*, 2006
ES663R	TACRAATARGAAACGRTCTCTCCAACG	Korall *et al.*, 2006
ESRBCL1361R	TCAGGACTCCACTTACTAGCTTCACG	Korall *et al.*, 2006
atpB		
ATPB672F	TTGATACGGGAGCYCCTCTWAGTGT	Wolf, 1997
ATPB1163F	ATGGCAGAATRTTTCCGAGATRTYA	Wolf, 1997
ATPB1419F	CRACATTTGCACATYTRGATGCTAC	Wolf, 1997
ATPB1592R	TGTAACGYTGYAAAGTTTGCTTAA	Wolf, 1997
ATPB609R	TCRTTDCCTTCRCGTGTACGTTC	Pryer *et al.*, 2004
ATPE384R	GAATTCCAAACTATTCGATTAGG	Pryer *et al.*, 2004

AM184112, respectively. All *rbcL* and *atpB* sequences for the other 62 taxa included here were taken from Pryer *et al.* (2004). No insertions or deletions (indels) were required to align *rbcL* or *atpB*.

Phylogenetic analyses.—The *rbcL* and *atpB* data sets were analysed with a Bayesian Markov Chain Monte Carlo approach (B/MCMC) using the parallel version of MrBayes 3.0B4 (Ronquist and Huelsenbeck, 2003), and equally weighted maximum parsimony (MP) with PAUP* version 4.0 beta 10 (Swofford, 2002). All analyses were performed on the CSEM/OIT high-performance, shared computing cluster at Duke University (Durham, North Carolina, USA). Trees were rooted with three lycopods: *Huperzia* Bernh., *Selaginella* Pal. Beauv., and *Isoetes* L. (Pryer *et al.*, 2004).

Nucleotide substitution models for the Bayesian analyses were chosen using MrModeltest 2.2 (a modified version of Modeltest 3.6; Posada and Crandall, 1998). Each gene was treated as a single partition (following Pryer *et al.*, 2004), and for both *rbcL* and *atpB* the general time reversible model (GTR) with a gamma distribution of substitution rates (Γ) and a proportion of invariant sites (I) was suggested based on the Akaike information criterion (AIC). Separate analyses of the two datasets were run for 3 million generations, on six parallel chains, with the temperature parameter (for heating the chains) set to 0.1. The sampled values for different parameters were examined using MrBayes and Tracer v. 1.2.1 (Rambaut and Drummond, 2005) to see if the parameters had converged. We also checked for a proper mixing of the chains. For each analysis every 1000th tree was sampled and, after analysing parameter values, 300 trees were discarded as "burn-in". Each analysis was repeated four times to ascertain that apparent stationarity was reached. The majority rule consensus trees from the replicates were compared and no differences were found. Trees from each analysis were then pooled (a total of 10800 trees, excluding those discarded as burn-in) before a majority rule consensus tree was calculated. In our Bayesian analyses, we consider branches with a posterior probability (PP) of 1.00 as well supported, a PP between 0.95–0.99 as moderately supported, and a PP of <0.95 as low support.

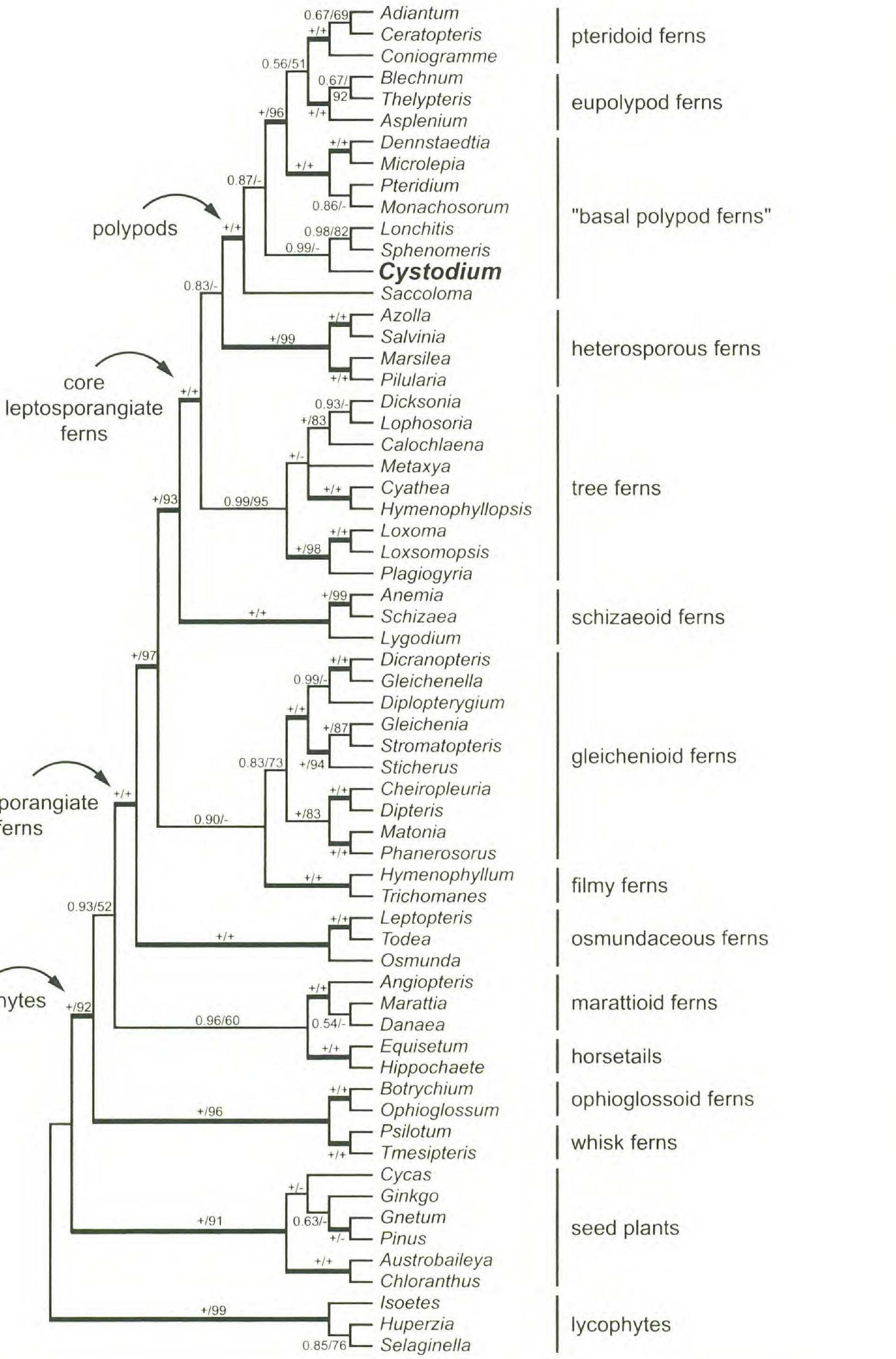

FIG. 1. 50% majority-rule consensus tree resulting from Bayesian (B/MCMC) analyses of the combined (*rbcL* and *atpB*) data set. Numbers on branches denote support values from Bayesian

MP analyses for each data set included a heuristic search for the most parsimonious trees with 1000 random-sequence-addition replicates, and TBR branch swapping. Support for nodes was calculated by a bootstrap analysis, with 3000 replicates, each with 10 random-sequence-addition replicates, and TBR branch swapping. In our MP analyses, we considered branches with a bootstrap percentage (BP) of $\geq$90% as well-supported, 70–89% as moderately supported, and <70% as weakly supported.

Combinability of rbcL *and* atpB *data sets.*—To evaluate combinability of data sets, the resultant topologies from each of the single-gene analyses were compared and examined for potential conflicts. Comparisons were made between topologies produced by the same analytical method, i.e., B/MCMC was compared with B/MCMC, and MP with MP. Incongruence supported by a MP bootstrap percentage of 70 or higher, or a Bayesian posterior probability of 0.99 or higher, was considered a conflict. Because no conflicts were observed, the two data sets were combined into a single data set.

Analyses of the combined data set.—The combined data set was analysed with settings as for the separate data sets. In the B/MCMC analyses, the two genes were each treated as one partition (i.e., a total of two partitions) and each was assigned the GTR + Γ + I model of nucleotide substitution (as in the separate data set analyses).

Results

Tree statistics.—All three data sets (*rbcL, atpB*, and combined) included 63 taxa. The *rbcL* data set included 1320 characters of which 559 were parsimony informative, the *atpB* data set 1150 characters (562 parsimony informative), and the combined data set 2470 characters (1121 parsimony informative). In MP, the *rbcL* analysis yielded two most parsimonious trees, with a tree length of 4788 steps, found in one island; the *atpB* analysis yielded 47 trees of 4088 steps in one island; the combined analysis resulted in eight trees of 8895 steps found in two islands (trees not shown).

Conflicting topologies.—Conflicts in leptosporangiate fern relationships (i.e., incongruence supported by a BP $\geq$ 70% or PP $\geq$ 0.99) were not found when MP topologies from the separate analyses of *atpB* and *rbcL* were compared to each other, or when B/MCMC analyses were compared to each other. The only conflict found was when comparing between analytical methods *within* the combined analysis: in the tree fern clade, *Metaxya* is sister to the other tree ferns in MP (BP = 89%), but is nested within the clade with strong support in B/MCMC (PP = 1.00).

←

(posterior probability; PP) and maximum parsimony (bootstrap percentages; BP) analyses. A plus (+) represents a PP = 1.00, or BP = 100%. A dash (–) represents a bootstrap percentage <50%. Thickened branches are well supported (PP = 1.00, BP $\geq$ 90%). Names of clades follow Pryer *et al.* (2004) and Schneider *et al.* (2004).

Phylogenetic relationships of leptosporangiate ferns.—Because the resultant topologies for leptosporangiate relationships from each of the separate gene analyses were not in conflict with one another, the phylogenetic relationships presented here are based on analyses of the combined data set (Fig. 1).

Cystodium, the focus of our study, is resolved as part of the polypod fern clade with high support (PP = 1.00, BP = 100%; Fig. 1). Within polypods, there is a weakly supported basal split (PP = 0.87, BP < 50%) with *Saccoloma* Kaulf. sister to the rest. The subsequent divergence yields a clade where *Cystodium* is sister to *Lonchitis* L. + *Sphenomeris* Maxon (PP = 0.99, BP < 50%), and together these taxa are sister to a large clade that includes the well-supported pteridoid, eupolypod, and a more exclusive group of basal polypod ferns (PP = 1.00, BP = 96%).

The polypods are included in the core leptosporangiate ferns (PP = 1.00, BP = 100%) together with the tree ferns (PP = 0.99, BP = 95%) and the heterosporous ferns (PP = 1.00, BP = 99%). The core leptosporangiate ferns are part of the well-supported leptosporangiate ferns (PP = 1.00, BP = 100%) as sister to the strongly supported schizaeoid ferns (PP = 1.00, BP = 100%). Filmy ferns and gleichenioid ferns group together with low support (PP = 0.90, BP < 50%) and the osmundaceous ferns are strongly supported as sister to the rest of leptosporangiate ferns (PP = 1.00, BP = 97%; Fig. 1).

Discussion

Conflicting topologies.—In the combined analysis, the conflicting positions of *Metaxya* within the tree ferns between analytical methods (MP vs. B/MCMC) was also recovered in a study on tree ferns (Korall *et al.*, 2006) and is discussed there as likely being due to a long branch attraction artefact (Felsenstein, 1978) in the MP analysis.

Phylogenetic relationships of leptosporangiate ferns, Cystodium *in particular.*—The overall leptosporangiate fern relationships shown in Fig. 1 are not in conflict with the results of Pryer *et al.* (2004). Our results clearly demonstrate that *Cystodium* is not a tree fern—it is a polypod (Fig. 1). *Cystodium* is likely closely related to *Sphenomeris* and *Lonchitis* (or perhaps *Saccoloma*), and is not included in the well-supported and species-rich clade that includes the pteridoid, eupolypod, and a more exclusive group of basal polypod ferns (clade names sensu Schneider *et al.*, 2004).

Several morphological and anatomical studies have revealed distinctive differences between *Cystodium* and other dicksonioid genera (Sen and Mittra, 1966; Gastony, 1981; Nishida, 1984; Croft, 1986; Tryon and Lugardon, 1991), leading some authors to suggest a close affinity between *Cystodium* and *Saccoloma* and/or *Dennstaedtia* Bernh., for example.

Sen and Mittra (1966) listed several features to separate *Cystodium* from *Dicksonia* (including receptacle shape, cortex structure, and orientation of subsidiary and guard-mother cells), but they nevertheless considered it to be part of the dicksonioid-cyathaeoid group.

Their conclusion was contradicted by Nishida (1984) and Croft (1986) who thoroughly studied the stipe vasculature of *Cystodium* and showed major differences with other dicksonioid genera. In cross section the stipe vasculature of *Cystodium* consists of several strands that unite to first form two bilateral branched arcs, and then, closer to the lamina, two dorsiventral, branched arcs. In *Dicksonia* several vascular bundles form one abaxial and two lateral arcs, which unite into a single incurved (U-shaped) strand. Other dicksonioid genera have vascular strands similar to *Dicksonia*. This led Croft (1986) to transfer *Cystodium* to a separate, monotypic family, Cystodiaceae, and to suggest that *Cystodium* be compared to "other groups with 2-lipped indusia and hairy rhizomes, such as the Dennstaedtiaceae" (in the broad sense, e.g., following Kramer, 1990).

Croft (1986) also noted that the annulus of *Cystodium* sporangia is not fully oblique and continuous, as is typical of other dicksonioid genera. It should be noted, however, that there is no general agreement on how to interpret the tree fern annulus; one morphological cladistic study considers the annulus to be oblique (Stevenson and Loconte, 1996), whereas another treats it as vertical to slightly oblique (i.e., as in the polypods; Pryer *et al.*, 1995).

Further supporting a relationship with the Dennstaedtiaceae *s.l.* is the striated perine of the spores. *Cystodium* shares this spore ornamentation with *Saccoloma* and some species of *Dennstaedtia* (Gastony, 1981; Tryon and Lugardon, 1991).

In summary, several morphological and anatomical studies have suggested significant differences between *Cystodium* and other genera traditionally assigned to Dicksoniaceae. In addition, these studies propose some similiarities to Dennstaedtiaceae *s.l.* In order to gain a better understanding of the precise phylogenetic position of *Cystodium*, the taxon sampling of early diverging lineages of polypods will need to be greatly expanded. Until then, we advocate the tentative inclusion of *Cystodium* in Lindsaeaceae, following a new classification for extant ferns (Smith *et al.*, 2006), as it is certainly not a member of Dicksoniaceae.

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